Functional evidence implicating FOXL2 in non syndromic premature ovarian failure and in the regulation of the transcription factor OSR2.

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Abstract

Background: *FOXL2* encodes a *forkhead* transcription factor whose mutations are responsible for the Blepharophimosis-Ptosis-Epicanthus inversus Syndrome (BPES), involving craniofacial/palpebral abnormalities often associated with premature ovarian failure (POF).

Results: We describe a FOXL2 variant (p.Gly187Asp) in a case of POF without BPES. The subcellular localization of FOXL2-G187D was normal but its transactivation capacity tested on two reporter promoters potentially relevant to the ovary was significantly lower than that of normal FOXL2. However, FOXL2-G187D was able to strongly activate a reporter construct driven by the promoter of *Osr2* (*odd-skipped related 2* transcription factor), which we have suggested to be a crucial target of FOXL2 in the craniofacial region. This is compatible with the absence of BPES in our patient.

Conclusions: Our data provide evidence in favor of the implication of *FOXL2* variants in non-syndromic POF and confirm the regulatory interaction between FOXL2 and *OSR2* whose perturbation might contribute to the palpebral abnormalities observed in BPES patients.

Short Report

FOXL2 is a single-exon gene encoding a *forkhead* transcription factor[1] expressed in the ovarian somatic compartment and peri-ocular tissues[2]. In humans, its mutations cause the Blepharophimosis-Ptosis-Epicanthus inversus Syndrome (BPES), which involves craniofacial/palpebral abnormalities associated or not with premature ovarian failure (POF)[3].

Here, we study the effects of a FOXL2 variant detected in a POF case without BPES. Clinically, the 27 year-old patient of Tunisian origin, presented with secondary amenorrhea. She had a normal pubertal development and no history of diabetes, pelvic surgery, radiation or autoimmunity. Consanguinity and familial history of infertility were excluded. She had high FSH (45 IU/L; normal menopausal range: 25–160 IU/L) and LH (35 IU/L; normal menopausal range: 11.3–40 IU/L) levels and low E2 (20 pg/mL; normal menopausal range <55 pg/mL). Cytogenetic analysis showed a normal 46,XX karyotype. Pelvic and ultrasound examination showed normal uterus and ovaries with several small follicles. The patient had none of the typical features of BPES (*A signed informed consent is available from the JMG website*).

Direct sequencing of the *FOXL2* coding region (performed as previously described[4]) revealed the presence of the heterozygous variant c.560G>A, inherited from her father, leading to the amino acid substitution p.Gly187Asp (FOXL2-G187D) in a highly conserved segment, C-terminal to the *forkhead* DNA-binding domain (Figure 1). This variant was absent in 110 control chromosomes. To assess the potential deleterious effect of the amino acid change we used the SIFT software (http://blocks.fhcrc.org/sift/SIFT.html). SIFT uses protein sequence conservation data to calculate the probability of a substitution of being deleterious. Scores lower than 0.05 suggest a potential

pathogenicity. The score of p.Gly187Asp, obtained using the alignment shown in Figure 1, was 0. The substitution p.Gly187Asp implies an important change in polarity. Glycine is a small neutral amino acid whereas aspartate is acidic and negatively charged at pH 7. Interestingly, Tyr186 is predicted to be phosphorylatable (http://www.cbs.dtu.dk/services/NetPhosK/). Therefore, we hypothesize that the negative charge of the side chain of aspartate might interfere with the post-translational modification process or mimic the modification itself, perturbing ovarian-specific protein interactions and/or regulations.

To assess subcellular localization and transcriptional activity of FOXL2-G187D, we performed transfection experiments and luciferase reporter assays as previously described[5,6]. The FOXL2-G187D variant fused to the green fluorescent protein displayed a typical nuclear staining, indistinguishable from that of normal FOXL2. From a functional perspective, FOXL2-G187D was found to activate the promoter of FOXL2 itself and the FOXL2-specific artificial promoter 2xFLRE-luc (containing 2 FOXL2 response elements upstream of a minimal CMV promoter)[6], though significantly less strongly than the normal protein. The p.Ile84Ser mutant, responsible for a type I BPES[7], was used as a negative control and was, as expected, unable to transactivate our luciferase reporters. We have recently suggested that a positive feedback loop of FOXL2 (which activates its own promoter) might be important in response to oxidative stress in the ovary[8]. Thus, we hypothesize that a lowered transactivation capacity of FOXL2-G187D on ovarian targets might explain the ovarian phenotype in our patient (Figure 2 A, B). However, the effects of this variant is expected to be promoter-dependent, as previously shown for other mutants[5,6], and as shown below for the third reporter promoter that we tested.

We have previously suggested that, in the craniofacial region, the encoding *odd-skipped related 2* transcription factor (*OSR2*) should be a crucial target of FOXL2[9]. *Osr2* is, as *Foxl2*, highly expressed in the murine periocular mesenchyma[10,11]. Moreover, as described for *Foxl2*$^{-/-}$ mice, *Osr2*$^{-/-}$ mutant neonates exhibit open eyelids with similar abnormalities[10,11]. Unfortunately, no data concerning the *Osr2*$^{-/-}$ ovary are available yet[10,12]. In agreement with our hypothesis and with the absence of an eyelid phenotype in our patient (and in her mutation-carrier father), we found that FOXL2-G187D was able to strongly activate a reporter construct driven by the *Osr2* promoter (p*OSR2)* described by Kawai et al 2005[12]. Coherently, the FOXL2-I84S mutant found in a BPES I patient failed to transactivate p*Osr2* (Figure 2 C). Since no second BPES locus has been reported thus far, it is unlikely that mutations in *OSR2* itself might contribute directly to BPES. However, regulatory mutations perturbing the interaction FOXL2-p*OSR2* are worth being screened in BPES patients with normal *FOXL2*.

The fact that FOXL2-G187D may be either normal or hypomorph suggests that it can behave as a susceptibility variant whose activity might be shifted up or down by modifier genes, leading to normal ovarian function or POF, respectively. Given our functional evidence, it would be interesting to formally assess the association between p.Gly187Asp and POF.

A previous mutation screening has detected a 30 bp deletion removing 10 out of 14 alanines of the FOXL2 polyAlanine tract (i.e. FOXL2-Ala4) and the substitution p.Tyr258Asn in two POF patients[13,14]. Interestingly, neither mutation was identified in 200 control chromosomes. However, the fact that the p.Tyr258Asn variant was maternally inherited and that the patient carrying the FOXL2-Ala4 variant spontaneously conceived and delivered two babies did not

allow a formal causal implication of these alterations in absence of functional assays at the time of these studies. Both variants might display incomplete penetrance or lead, at least, to partial fertility recovery after appropriate treatments. Thus, it would be interesting to assess the functional impact for both alterations.

Taken together, our results provide functional evidence in favor of the implication of *FOXL2* variants in non-syndromic POF and confirm the regulatory interaction between FOXL2 and *OSR2*, whose perturbation might contribute to the palpebral phenotype in BPES. In our opinion, a more systematic genetic screening of FOXL2 mutations is of interest in non-syndromic POF, to improve genetic counseling and to better understand the molecular etiology of this frequent pathology.

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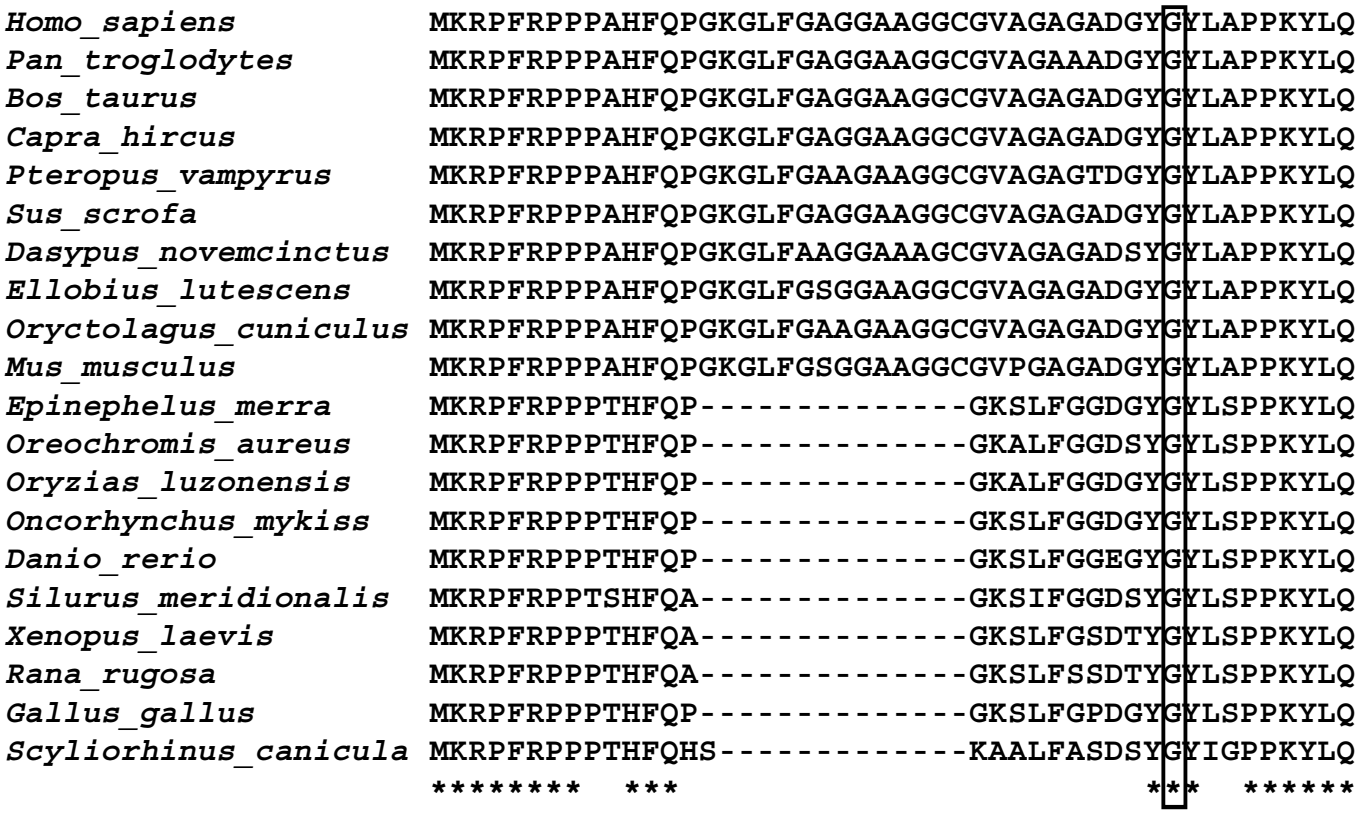
Figure legends.

Figure 1: FOXL2 sequence alignment around Gly187. The alignment involves sequences from vertebrates ranging from amphibian and fishes to mammals. The relevant Gly187 residue is highlighted within a frame. The immediately previous highly conserved Tyr/Y residue is predicted to be phosphorylatable. The negative charge of aspartate in FOXL2-Gly187Asp might interfere with the post-translational modification process or mimic the modification itself.

Figure 2. Luciferase assays using three reporter promoters. For transfections, we used granulosa-like KGN cells and each experiment was performed in 5 replicates. The variant p.Ile84Ser was obtained by fusion PCR as previously described[5,6]. A) Activity on the FOXL2-specific artificial promoter 2xFLRE-luc (containing 2 FOXL2 response elements upstream of a minimal CMV promoter)[6], B) Activity on the promoter of FOXL2 itself and C) Activity on p*Osr2*. In all cases ANOVAs were significant ($p<0.001$). Post-hoc Tuckey HSD tests showed that the mutant p.Ile84Ser always behaved no differently than the empty control vector, while G187D had a significant activity, through lower than the wild-type/WT protein for 2xFLRE-luc and pFOLX2-luc (A,B). Interestingly, in the case of pOsr2-luc (C), G187D had an activity even stronger than the WT. In all cases, p-values were at least < 0.05.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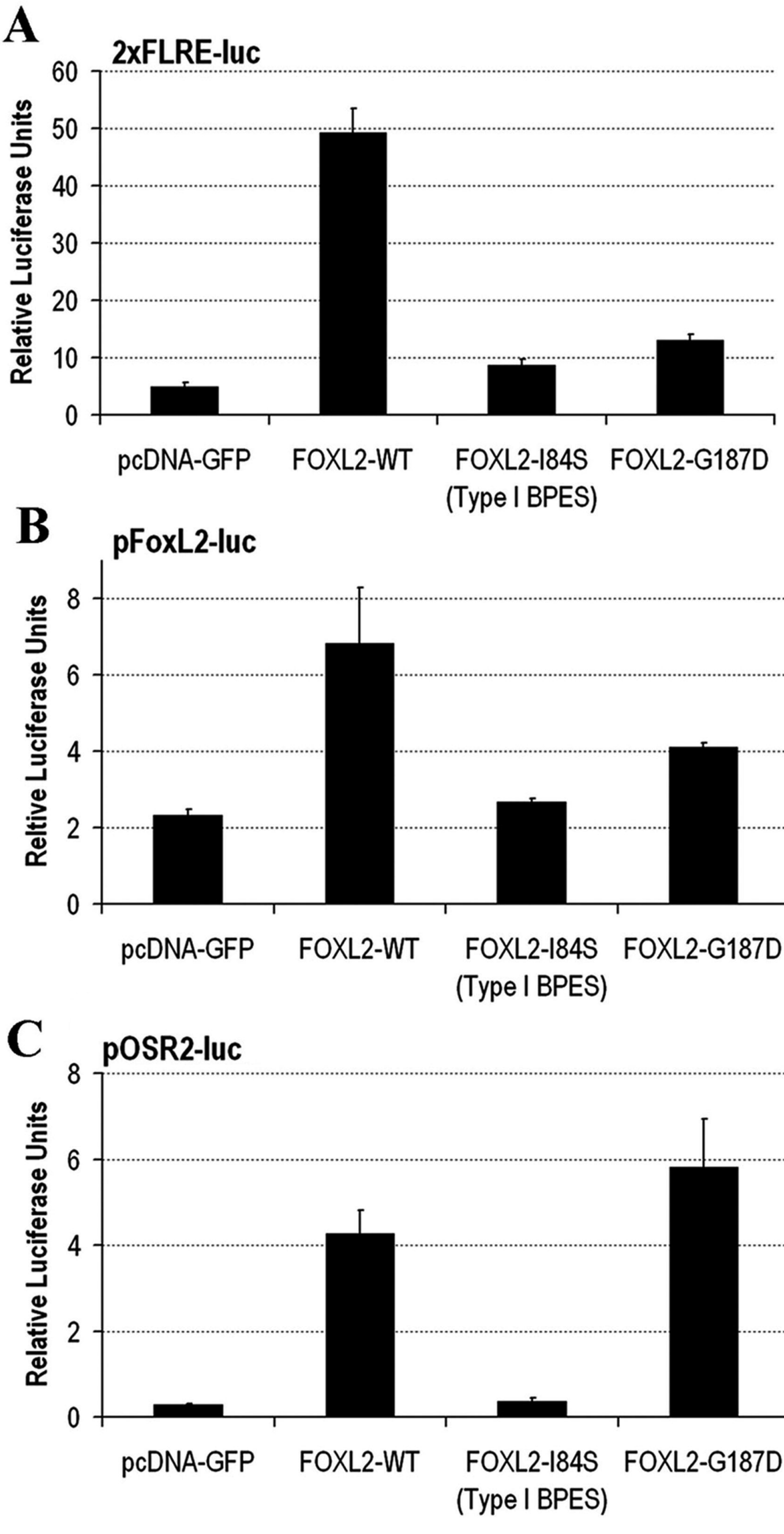
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